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(71) Applicants and
(72) Inventors: **COOPER, Garth, James, Smith** [NZ/NZ]; 7 Marine Parade, Herne Bay, Auckland (NZ). **BUCHANAN, Christina, Maree** [NZ/NZ]; 92 Hadfield Street, Birkenhead, Auckland (NZ).

(74) Agents: **BENNETT, Michael, Roy** et al.; West-Walker Bennett, Mobil on the Park, 157 Lambton Quay, Wellington (NZ).

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(54) Title: PEPTIDE HAVING PREPTIN FUNCTIONALITY

(57) Abstract: The invention relates to a bioactive mammalian peptide. In particular, it relates to a peptide secreted by the pancreatic islet β-cell that stimulates insulin secretion, termed preptin. Preptin analogs, pharmaceutical compositions which contain preptin or its analogs and their use as medicaments are *inter alia* also provided.

PEPTIDE HAVING PREPTIN FUNCTIONALITY

This invention relates to a bioactive peptide. In particular, it relates to a peptide secreted by the pancreatic islet β-cell that stimulates insulin secretion.

BACKGROUND

Pancreatic islet β-cells play a major regulatory role in physiology, mainly through their secretion of insulin, a peptide hormone which exerts profound effects on intermediary metabolism (Draznin *et al* (1994)). A second β-cell hormone, amylin, may also contribute to β-cell regulatory function through its actions on insulin secretion and tissue insulin sensitivity (Cooper, G (1994); Hettiarachchi *et al* (1997)).

In islet β-cells, hormones are packaged in secretory granules, which undergo regulated release in response to signals such as fuels (eg. glucose, amino acids) or neurohormonal stimuli. These granules contain dense cores rich in insulin and Zn, while smaller amounts of insulin C-peptide, amylin, proinsulin, chromogranin-derived peptides, proteases and other proteins are found in the granule matrix (Hutton, J (1989)).

What the applicants have now determined is that pancreatic islet β-cells secrete yet a further regulatory peptide. The applicants have further determined that this peptide enhances glucose-mediated insulin secretion.

It is generally towards this peptide, which the applicants have termed preptin, that the present invention is directed in its various aspects.

SUMMARY OF THE INVENTION

Accordingly, in a first aspect the present invention provides the peptide preptin or an analog thereof.

By "preptin", the applicants mean a peptide of 34 amino acids, the sequence of which is as follows:

Asp Val Ser Thr R_1 R_2 R_3 Val Leu Pro Asp R_4 Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe R_5 R_6 Asp Thr Trp R_7 Gln Ser R_8 R_9 Arg Leu

wherein:

R_1 is Ser or Pro;

R_2 is Gln or Pro;

R_3 is Ala or Thr;

R_4 is Asp or Asn;

R_5 is Gln or Lys;

R_6 is Tyr or Phe;

R_7 is Arg or Lys;

R_8 is Ala or Thr; and

R_9 is Gly or Gln,

or an analog thereof.

In one embodiment, the invention provides human preptin having the amino acid sequence:

Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr Trp Lys Gln Ser Thr Gln Arg Leu.

In another embodiment, the invention provides rat preptin having the amino acid sequence:

Asp Val Ser Thr Ser Gln Ala Val Leu Pro Asp Asp Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Lys Phe Asp Thr Trp Arg Gln Ser Ala Gly Arg Leu.

In yet another embodiment, the invention provides mouse preptin having the amino acid sequence:

Asp Val Ser Thr Ser Gln Ala Val Leu Pro Asp Asp Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr Trp Arg Gln Ser Ala Gly Arg Leu.

The amino acid sequence corresponds to Asp_{69}-Leu_{102} of the proIGF-II E-peptide in each mammal.

In still a further aspect, the present invention provides a polynucleotide which encodes preptin or an analog thereof.

In another aspect, the invention provides a vector or cell-line which includes a polynucleotide which encodes preptin or an analog thereof and which is capable of expressing preptin or said analog.

Preptin salts, which are preferably physiologically acceptable, are also provided.

In a further aspect, the invention further provides a pharmaceutical composition which comprises preptin or an analog thereof, or preptin salts.

In still a further aspect, the invention provides a method of stimulating insulin secretion for a therapeutic or prophylactic purpose which comprises the step of administering to a patient in need of such therapy or prophylaxis an effective amount of preptin or an analog thereof.

In yet a further aspect, the invention provides the use of preptin or an analog thereof or a salt thereof in the preparation of a medicament, particularly for stimulating insulin secretion.

In still a further aspect, the invention provides a method of modulating glucose mediated insulin secretion which comprises the step of administering to a patient an effective amount of preptin, a preptin analog, a preptin agonist or a preptin antagonist.

In yet further embodiments, the invention provides antibodies which bind preptin or its analogs, assays which employ such antibodies and assay kits which contain such antibodies.

The above summary is not exhaustive. Other aspects of the invention will be apparent from the following description, and from the appended claims.

DESCRIPTION OF THE DRAWINGS

Although the invention is broadly as defined above, it will also be understood that it includes embodiments of which the description provided below gives examples. In addition, the invention will be better understood by reference to the accompanying drawings in which:

Figure 1 shows purification and characterisation of preptin. a) assays for marker proteins indicating the localisation of organelles from βTC6-F7 cells within the continuous OptiPrep gradient; granule core (insulin), granule matrix (amylin), lysosomes (aryl sulphatase), mitochondia (citrate synthase). b) Granule proteins purified by RP-HPLC. The indicated peak (hatched) was collected and further purified. c) Purity and mass ($M + H^+$) of the major peptide from the hatched peak confirmed by MALDI-TOF MS. d) RP-HPLC profile from the Lys-C digest of the peptide purified from the hatched peak. 1:NH_2-terminal fragment; 2: COOH-terminal fragment; 3: undigested peptide. e) Structure of mouse preptin as determined by sequencing of Lys-C-derived peptides from (d); NH_2-terminal fragment: normal font; COOH-terminal fragment: italicised-bold, and its localisation in a segment of murine proIGF-II E-peptide shown. Domains of proIGF-II (B, C, A, D, E) are indicated. Recognised cleavage site at Arg_{68} is indicated in bold, while putative dibasic motifs are shown as discontinuous lines.

Figure 2 shows cellular preptin secretion. a) Preptin RIA standard curve. b) RIA characterisation of preptin-like immunoreactive material (PLIM) in RP-HPLC fractions of 24-h βTC6-F7 conditioned medium and intra-granular fractions from Figure 1b. c) MALDI-TOF MS of the major PLIM containing fraction secreted from βTC6-F7 cells. Peak corresponds to murine preptin ($M + H^+$) with 0.07% error.

Figure 3 shows the effects of preptin on insulin secretion. a) Preptin-mediated insulin secretion from βTC6-F7 cells. Graph illustrates increments in insulin concentration above basal (0 added preptin). b) preptin-mediated insulin secretion from isolated perfused rat pancreas. Points are mean $\pm$ sem (duplicate analyses; n=4 pancreases for each curve). Area under curve (second phase of insulin secretion P = 0.03 unpaired 2-tailed t-test).

Figure 4 shows the immunohistochemistry of murine pancreas. Pancreas harvested from adult FVB/n mice was sectioned and stained with haematoxylin and polyclonal rabbit antisera using immunoperoxidase-conjugated goat-anti-rabbit second antibody. Panels are: **a**, anti-insulin antiserum (1:40); **b**, anti-preptin antiserum, (1:40); **c**,**d**, anti-preptin antiserum (1:40) pre-incubated for 30 min with synthetic rat preptin at **c**, 1 $mg.ml^{-1}$, **d**, 5 $mg.ml^{-1}$. Bar = 100 μm.

Figure 5 shows the RIA characterisation of preptin-like immunoreactive material (PLIM) in RP-HPLC fractions from rat islets or βTC6-F7 granule fractions (standard; Fig. 1b).

Figure 6 shows preptin and insulin co-secretion from βTC6-F7 cells and isolated rat islets. **a,b** Glucose-mediated co-secretion of preptin with insulin from **a**, βTC6-F7 cells and **b**, isolated rat islets.

Figure 7 shows the effects of preptin on insulin secretion. **a**, **b**, Purity and mass of purified **a**, rabbit anti-rat preptin γ-globulin and **b**, non-immune rabbit γ-globulin. 1: light chain IgG, $M + H^+$; 2: whole IgG, $M + 4H^+$; 3: heavy chain IgG, $M + H^+$; 4: whole IgG, $M + 2H^+$; whole IgG, $M + H^+$. **c**, 1-min preptin-binding capacity of perfused anti-preptin γ-globulin at 35 $\mu g.ml^{-1}$, 37°C, pH 7.4 to simulate contact time, dilution, temperature and pH of the antibody perfusion experiments. **d**, Effect of infusion of anti-preptin γ-globulin or control (non-immune rabbit γ-globulin) on insulin secretion from glucose-stimulated (20 mM; square wave) isolated perfused rat pancreases. Each point is mean ± s.e.m. (duplicate analyses; $n = 5$ pancreases per curve). AUC (second phase of insulin secretion; $P = 0.03$, unpaired 1-tailed t-test).

DESCRIPTION OF THE INVENTION

As broadly defined above, the present invention is directed to a novel peptide which has been found in pancreatic islet β-cell granules. This peptide, preptin, has been determined to stimulate glucose-evoked insulin secretion.

In summary, preptin was identified using a single-step density-gradient centrifugal method to purify secretory granules from cultured murine βTC6-F7 cells with purity being confirmed by marker-protein analysis (Figure 1a). Insulin was used to track purification of granule-cores, whereas amylin, which is present in the granule-matrix (Johnson, K (1988)), was measured to verify granule-membrane integrity (Figure 1a). Soluble granule components were then separated using reversed-phase HPLC (A_{214}; Figure 1b). Peptide-identity was determined by mass spectrometry and NH_2-terminal amino-acid sequencing. Major peaks contained murine insulins-I and -II and C-peptides-I and -II (Figure 1a). No non-β-cell peptides were detected and the

molar ratio of amylin:insulin (1:23) and mouse insulin I:mouse insulin II (1:3) were equivalent to those of physiological β-cells (Cooper (1994); Linde (1989)).

A major peak eluting immediately prior to insulin-I was found to contain a previously unknown peptide (Figure 1b). This was purified to homogeneity and had a molecular mass of 3950 Da (Figure 1c). The molecule was digested with a lysine-specific protease, and the resulting peptides separated by RP-HPLC (Figure 1d) prior to complete NH_2-terminal protein sequencing. The complete sequence confirmed that the molecule contained 34-amino acids, which corresponded to Asp_{69}-Leu_{102} of murine proIGF-II E-peptide (Figure 1e). This peptide is mouse preptin.

Preptin is flanked NH_2-terminally by a recognised Arg cleavage-site, and COOH-terminally by a putative dibasic (Arg-Arg) cleavage motif (Bell *et al.*, (1985)) (Fig. 1e). These residues are highly conserved between species, and are likely to serve as post-translational processing signals.

While others have shown the existence of different proIGF-II E-peptide-derived peptides in cell culture medium and various mammalian biological fluids (Hylka *et al.*, (1985), Daughaday *et al* (1992) and Liu *et al.*, (1993)), none have identified one that is equivalent to preptin.

The amino acid sequence of mouse preptin is as follows:

Asp Val Ser Thr Ser Gln Ala Val Leu Pro Asp Asp Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr Trp Arg Gln Ser Ala Gly Arg Leu

The equivalent amino acid sequences for human and rat preptin are, respectively:

Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr Trp Lys Gln Ser Thr Gln Arg Leu; and

Asp Val Ser Thr Ser Gln Ala Val Leu Pro Asp Asp Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Lys Phe Asp Thr Trp Arg Gln Ser Ala Gly Arg Leu.

Preptin is encoded by polynucleotides having the following nucleotide sequences:

gacgtgtcgacccctccgaccgtgcttccggacaacttccccagataccccgtgggcaagttcttccaatatga cacctggaagcagtccacccagcgcctg (human)

gacgtgtctacctctcaggccgtacttccggacgacttccccagataccccgtgggcaagttcttcaaattcgac acctggagacagtccgcgggacgcctg (rat)

gacgtgtctacctctcaggccgtacttccggacgacttccccagataccccgtgggcaagttcttccaatatgac acctggagacagtccgcgggacgcctg (mouse)

Preptin may be generated by synthetic or recombinant means. For example, to be prepared synthetically, preptin may be synthesised using any of the commercially available solid phase techniques such as the Merryfield solid phase synthesis method, where amino acids are sequentially added to a growing amino acid chain (see Merryfield, *J. Am. Soc.* 85:2146-2149 (1963)). Equipment for automative synthesis of peptides is also commercially available from suppliers such as Perkin Elmer/Applied Biosystems, Inc and may be operated according to the manufacturers instructions.

Preptin may also be produced recombinantly by inserting a polynucleotide (usually DNA) sequence that encodes the protein into an expression vector and expressing the peptide in an appropriate host. Any of a variety of expression vectors known to those of ordinary skill in the art may be employed. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA molecule which encodes the recombinant peptides. Suitable host cells include prokaryotes, yeasts and higher eukaryotic cells.

Standard techniques for recombinant production are described for example, in Maniatis *et al*, Molecular Cloning - A Laboratory Manual, Cold Spring Harbour Laboratories, Cold Spring Harbour, New York (1989).

Vectors and/or cells lines which express preptin have utility in their own right and also form part of the invention.

Analogs of preptin and of its encoding polynucleotides are also within the scope of the present invention. Such analogs include functional equivalents of preptin and of the polynucleotides described above.

In terms of preptin itself, functional equivalents include all proteins which are immunologically cross-reactive with and have substantially the same function as preptin. That equivalent may, for example, be a fragment of preptin containing from 6 to 33 amino acids (usually representing a C-terminal truncation) and including a preptin active site or sites, a substitution, addition or deletion mutant of preptin, or a fusion of preptin or a fragment or a mutant with other amino acids.

The six amino acids forming the smallest fragment can be from any part of the sequence, provided they are consecutive in that sequence and fulfil the functional requirement. It is of course also possible (and expressly contemplated) that the bioactive peptide include any one of those hexapeptides, or indeed be or include any heptapeptide, octapeptide, nonapeptide, or decapeptide from the sequence.

Peptides which are, or include a hexapeptide, heptapeptide, octapeptide, nonapeptide or decapeptide from human preptin are particularly preferred.

Variations in the residues included in the peptide are also both possible and contemplated. For example, it is possible to substitute amino acids in a sequence with equivalent amino acids using conventional techniques. Groups of amino acids known normally to be equivalent are:

(a) Ala Ser Thr Pro Gly;
(b) Asn Asp Glu Gln;
(c) His Arg Lys;
(d) Met Glu Ile Val; and
(e) Phe Tyr Trp.

Additions and/or deletions of amino acids may also be made as long as the resulting peptide is immunologically cross-reactive with and has substantially the same function as preptin.

Equivalent polynucleotides include nucleic acid sequences that encode proteins equivalent to preptin as defined above. Equivalent polynucleotides also include nucleic acid sequences that, due to the degeneracy of the nucleic acid code, differ from native polynucleotides in ways that do not effect the corresponding amino acid sequences.

A prediction of whether a particular polynucleotide or polypeptide is equivalent to those given above can be based upon homology. Polynucleotide or polypeptide sequences may be aligned, and percentage of identical nucleotides in a specified region may be determined against another sequence, using computer algorithms that are publicly available. Two exemplary algorithms for aligning and identifying the similarity of polynucleotide sequences are the BLASTN and FASTA algorithms. The similarity of polypeptide sequences may be examined using the BLASTP algorithm. Both the BLASTN and BLASTP software are available on the NCBI anonymous FTP server (ftp://ncbi.nlm.nih.gov) under /blast/executables/. The BLASTN algorithm version 2.0.4 [Feb-24-1998], set to the default parameters described in the documentation and distributed with the algorithm, is preferred for use in the determination of variants according to the present invention. The use of the BLAST family of algorithms, including BLASTN and BLASTP, is described at NCBI's website at URL http://www.ncbi.nlm.nih.gov/BLAST/newblast.html and in the publication of Altschul, Stephen F, *et al* (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25:3389-3402. The computer algorithm FASTA is available on the Internet at the ftp site ftp://ftp.virginia.edu.pub/fasta/. Version 2.0u4, February 1996, set to the default parameters described in the documentation and distributed with the algorithm, is preferred for use in the determination of variants according to the present invention. The use of the FASTA algorithm is described in the W R Pearson and D.J. Lipman, "Improved Tools for Biological Sequence Analysis," *Proc. Natl. Acad. Sci. USA 85:*2444-2448 (1988) and W.R. Pearson, "Rapid and Sensitive Sequence Comparison with FASTP and FASTA," *Methods in Enzymology 183*:63-98 (1990).

Analogs according to the invention also include the homologues of preptin from species other than human, rat or mouse. Such homologues can be readily identified using, for example, nucleic acid probes based upon the conserved regions of the polynucleotides which encode human, rat and mouse preptin.

Preptin or its analogs can also be present in various degrees of purity. Preferably, the preptin/analog component makes up at least 50% by weight of the preparation, more preferably at least 80% by weight, still more preferably at least 90% by weight, still more preferably at least 95% by weight and yet more preferably at least 99% by weight. It is however generally preferred that, for pharmaceutical application, the preptin or analog be present in a pure or substantially pure form.

For administration to a patient, it is possible for preptin or preptin analogs to be used as such pure or substantially pure compounds. However, preptin or preptin analogs may also be presented as a pharmaceutical composition. Such compositions may comprise preptin or preptin analogs together with one or more pharmaceutically acceptable carriers therefor and optionally other therapeutic ingredients where desirable.

The carrier must be acceptable in the sense of being compatible with the preptin or preptin analog and not deleterious to the patient to be treated. Desirably, the composition should not include substances with which peptides are known to be incompatible.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the active ingredients into association with a carrier which constitutes one or more accessory ingredients.

The precise form the composition will take will largely be dependent upon the administration route chosen. For example, preptin or preptin analogs may be injected parenterally, eg. intravenously into the blood stream of the patient being treated. However, it will be readily appreciated by those skilled in the art that the route can vary, and can be intravenous, subcutaneous, intramuscular, intraperitoneal, enterally, transdermally, transmucously, sustained release polymer compositions (eg. a lactide polymer or co-polymer microparticle or implant), perfusion, pulmonary (eg. inhalation), nasal, oral, etc.

Compositions suitable for parenteral and in particular intravenous administration are presently preferred. Such compositions conveniently comprise sterile aqueous solutions of preptin or the preptin analog. Preferably, the solutions are isotonic with the blood of the patient to be treated. Such compositions may be conveniently prepared by dissolving the preptin or analog in water to produce an aqueous solution and rendering this solution sterile. The composition may then be presented in unit or multi-dose containers, for example sealed ampoules or vials.

One particularly preferred composition is preptin in a physiological buffer solution suitable for injection.

Compositions suitable for sustained release parenteral administrations (eg. biodegradable polymer formulations) are also well known in the art. See, for example, US Patent Nos. 3,773,919 and 4,767,628 and PCT Publication No. WO 94/15587.

It is also convenient for preptin to be converted to be in the form of a salt. Such a salt will generally be physiologically acceptable, and can be formed using any convenient art standard approach.

Preptin salts formed by combination of preptin with anions of organic acids are particularly preferred. Such salts include, but are not limited to, malate, acetate, propionate, butyrate, oxaloacetate, citrate, isocitrate, α-ketoglutarate, succinate, fumarate and trifluoroacetate salts.

The salts this formed can also be formulated into pharmaceutical compositions for therapeutic administration where this is desired.

Aspects of the invention will now be described with reference to the following non-limiting experimental section.

EXPERIMENTAL

Section A

Methods and Materials

Cell Culture

βTC6-F7 murine-pancreatic islet β-cells, passages 49-60, were cultured at 37°C in O_2:$CO_2$95:5 (v/v) in triple flasks in nDMEM (Gibco) supplemented with 15% heat-inactivated horse serum and 2.5% fetal bovine serum, and subcultured every 5 d by washing with PBS followed by trypsinization (2.5% Trypsin-EDTA). Each flask yielded approx 2.0 x 10^8 cells at 70% confluence.

Granule purification

β-cells at passages 55-60 from 8-12 triple flasks were harvested by trypsinization, yielding on average 2.5-4.0 ml of pure cells (1.6 - 2.4 x 10^9), which then were concentrated (1700 x g, 5 min), washed twice with PBS, and once with

Homogenisation Medium (0.3 M sucrose/10 mM MES K (Sigma)/1 mM K_2EGTA/1 mM Mg_2SO_4/pH 6.5), then homogenised on ice in the same medium at 1:5 (v/v). The cell suspension was homogenised by 20 passages through a ball-bearing homogeniser (7.87 x 10^{-5} cm clearance), then clarified by centrifugation (400 x g, 10 min), the pellet once re-homogenised and centrifuged, and the supernatants combined (final vol = 20 ml). Solutions (v/v) of 13% and 31% OptiPrep™ (Nycomed) were prepared by dilution with Homogenisation Medium, and 6 x 10 ml continous gradients (31%-13% OptiPrep) poured (Auto Densi-Flow II, Haakebuchler) into Ultra-Clear tubes (Beckman). Pelleted material was over-layered or under-layered, then ultracentrifuged (SW40 Ti/160,000 x g/16 h/4°C). Fractions with RI of 1.363-1.368, containing highest purity secretory granules, were collected, whereas mitochondria and lysosomes were isolated to fractions with RI > 1.371. Integrity of granule preparations was monitored using radioimmunoassays for insulin (crystalline granule core), amylin (granule matrix); purity by functional assays for aryl sulphatase (lysosomes) and citrate synthase (mitochondria); and total protein content using Bicinchoninic acid (Pierce).

RP-HPLC

Granule proteins were purified in two sequential RP-HPLC runs (A: 0.08% TFA v/v; B: 80% acetonitrile with 20% A; Applied Biosystems 140B/785A/112A system; Jupiter C18 RP column,250 x 2.0 mm (Phenomonex); 250-300 μl/min; A_{214}). Secretory granule material was initially centrifuged (16,000 x g, 20 min) before loading. An initial 15 min isocratic step was employed, and sequential 30s fractions collected from 19 min post-injection. Slightly different gradients were used sequentually to purify proteins; the first semi-purified granule proteins, whereas the second was slightly flatter, to increase resolution and purity.

Peptide sequence analysis

Purified peptides were identified by N-terminal sequence determination (automated Edman method; ABI Procise™) combined with accurate mass determination by MALDI-TOF MS. For complete sequence verification, purified mouse preptin isolates were cleaved using Lys-C (Boehringer Mannheim), and the resulting peptide fragments repurified by RP-HPLC.

MALDI-TOF mass spectrometry

Peptide molecule weights were determined by MALDI-TOF MS (Hewlett-Packard G2025A; 337 nm-emission nitrogen laser/150 μJ maximum output/3 ns

pulsewidth/30 kV ion acceleration potential) fitted with a 500 MHz digital oscilloscope (G2030AA, LeCroy) using an α-CHC matrix with recombinant human insulin (Novo Nordisk; $M + H^+$, 5808.66 Da; $M + 2H^+$, 2904.83) and somatostatin (Bachem; $M + H^+$, 1638.91, $M + Na^+$, 1660.90) mass standards. MS was performed under high vacuum (<1.0 μTorr) and data acquired (ChemStation; 0-20PS method positive polarity in the 0-20 kDa range) with external mass calibration in "single shots" mode. Accurate molecular weights of purified peptides were confirmed by interpolation with external mass standardisation.

Chemical synthesis of rat preptin

The sequences of rat and human preptin were determined by comparison with known predicted IGF-II sequences. Rat preptin was chemically synthesised (Auspep Pty, Australia), according to the predicted sequence, using Fmoc chemistry on an Advanced Chem Tech 396 Robotics Peptide Synthesiser starting with FmocLeu-Wang resin. The peptide was deprotected and cleaved from the resin with a solution of 92.5% TFA: 2.5% water:2.5% triisopropylsilane: 2.5% dithiothreitol for 3 h. The peptide was precipitated from the TFA solution by addition of diisopropyl ether and the precipitate dissolved in 30% acetonitrile: water, lyophilised, and purified by RP-HPLC. Purity was confirmed as >99% by analytical RP-HPLC (rat preptin eluted at 47%B), while MALDI-TOF MS validated the mass as 3932.4 Da ± 0.026%.

Preptin radioimmunoassay

Synthetic rat preptin was conjugated to the carrier, ovalbumin, using the single step glutaraldehyde method at pH 7.0, then used to raise polyclonal antisera in NZW rabbits. Preptin was ^{125}I-radiolabelled using the chloramine-T method, and [^{125}I]preptin (362 μCi/μg) purified by Sephadex G-10 chromatography (50 mM phosphate buffer, pH 7.5). An optimised RIA for preptin was then developed, with B/F separation by the PEG-assisted second antibody (goat-anti-rabbit method). This employed a final dilution of antiserum at 1: 10,000 (final assay dilution 1: 30,000) at an R/T value of 0:30; tracer at 8,000 cpm/tube; incubation times of 24h + 72h; and had an EC_{20} value of 344 pM preptin; EC_{80} of 39 pM; minimum detectable concentration of 11.2 ± 9.8 pM; and zero cross-reactivity with rodent (rat/mouse) insulins and amylin.

Cellular preptin secretion

Preptin secretion was studied in βTC6-F7 cells (passage #52), cultured otherwise as above in 24-well plates at 4 x 10^5 cells per well. Preptin stimulation was performed

after 3 d growth, at 80% confluence. Cells were washed twice in HEPES-buffered KRB before commencement of secretion studies, then preincubated for 1 h in 1 ml/well incubation buffer (0 mM glucose; 0.1% w/w Fraction V BSA (Sigma) dissolved in HEPES-KRB) 500 µl/well was then removed, and replaced with an equivalent volume of fresh incubation buffer containing various concentrations of glucose. After 2 h incubation (37°C), incubation medium was removed, cells washed thrice with PBS, then lysed with lysis buffer. Incubation supernatants and cell lysates were then assayed for insulin and preptin contents using the described RIAs. In separate experiments, time-dependent hormone secretion was also determined.

Characterisation of secreted preptin-like immunoreactive material (PLIM)

Since preptin is a cleavage product of the E-peptide of IGF-II, and other cleavage products from a similar region have been isolated from serum in the past (Hylka (1985); Daughaday (1992); Liu (1993)), quantitation by preptin RIA was insufficient to characterise the nature of the secreted and circulating peptide. A combined RP-HPLC/preptin RIA method was therefore developed to further characterise PLIM.2 ml aliquots of separated plasma from a human donor, and βTC6-F7 conditioned medium, were acidified with 0.1 ml of 4M acetic acid and applied to a C-18 Sep Pak (Waters, 1 ml volume) which had been pre-equilibrated with 10 ml of 100% methanol and 20 ml of 4% (v/v) acetic acid. The Sep Pak was washed with 20 ml of 4% acetic acid, before bound components were eluted with 2 ml of 0.1 M acetic acid in 70% methanol, and the final volume of eluate reduced to 150 µl by rotary evaporation. Eluates were then subjected to RP-HPLC as above, and corresponding fractions combined from multiple runs. Fractions likely to contain preptin and insulin were subjected to MALDI-TOF MS. All fractions were then made up to a volume of 350 µl with preptin assay buffer, then analysed by preptin and insulin RIAs. In order to compare profiles of immunoreactivety of these secreted products with the intragranular profile (Figure 1b), 10 µl samples from the initial RP-HPLC granule fractions were diluted to a final volume of 610 µl with preptin RIA buffer, and also assayed for insulin and preptin.

Rate of carbohydrate metabolism in isolated rat skeletal muscle

The β-cell hormones amylin and insulin modulate carbohydrate utilisation in peripheral tissues, including skeletal muscle. The ability of preptin to alter glucose uptake and incorporation into muscle glycogen was investigated using isolated incubated stripped soleus muscle as a model tissue. All animal methods were carried out with appropriate permission from the Institutional Animal Ethics

Committee. Male Wistar rats (200 $\pm$ 20 g) were housed in controlled conditions (20°C, 12 h light/dark cycle) and fed standard rat chow (Diet 86, NRM Tegel, Auckland) and water *ad libitum*. 18-h fasted rats were anaesthetised (45 mg/kg Pentobarbitone sodium) then sacrificed by cervical dislocation, and soleus mucles dissected under carboxygenated-KHB (O_2:$CO_2$95: 5 v/v), then incubated in nDMEM supplemented with various concentrations of insulin and preptin. Muscles were teased longitudinally into 3 equal strips with a final radius of approximately 1.5 mm [(U)^{14}C] D(+)-glucose (1 mCi/ml, Amersham) was diluted 1: 20 (v/v) in 70% ethanol to yield a final concentration of 0.5 µCi/10 µl. Actrapid® Recombinant Human Insulin (100 U/ml, Novo Nordisk) was diluted 1/1000 in 10 ml nDMEM. 60 µg rat preptin was dissolved in 1526 µl of nDMEM to a concentration of 10 µM, then further diluted in nDMEM to give stock solutions of 1 µM, 100 nM, 10 nM, 100 pM and 1 pM. Two different experimental paradigms were employed to determine whether preptin (i) stimulated the rate of glucose incorporation into glycogen, or (ii) acted as an antagonist of insulin-evoked glucose incorporation into glycogen.

Preptin antagonist incubation protocol

Four muscle strips were transferred into each of 9 flasks, which contained 10 ml of carboxygenated nDMEM, 0 (control) or maximally-effective insulin (23.7 nM), and various concentrations of preptin (10 fM, 100 fM, 1 pM, 0, 10 pM, 100 pM, 1 nM or 10 nM). Flasks were then equilibrated in a shaking water bath (30°C, 20 min), following which 10 µl of (0.5 µCi) D-[(U)^{14}C] glucose was added, at strict 1.5 min intervals. Muscle strips were then incubated for 120 min at 30°C under carbogen. After incubation, strips were removed from each flask at 1.5 min intervals, and blotted dry. They were then snap-frozen in liquid N_2, freeze-dried for 24 h in pre-weighed tubes, then strip dry-weights determined. Muscle strips were then solubilised in 250 µl or 60% KOH, incubated at 70°C for 45 min, then cooled before overnight precipitation at -20°C with ice-cold ethanol. Glycogen pellets were then prepared by centrifugation (9,000xg, 15 min, 0°C), pellets resuspended, and re-precipitated twice, before the supernatant was finally aspirated and glycogen pellets over-dried at 70°C for 2 h. incorporation of ^{14}C was then determined by scintillation counting.

Preptin agonist protocol

All methods were as described above, except that strips were incubated in the absence of insulin (except for the positive control, at 23.7 nM) and final preptin concentrations of 0, 0.1, 1, 10 and 100 nM.

Effect of preptin on insulin secretion

Insulin and amylin are known to modulate β-cell insulin secretion via presumed autocrine mechanisms. The effect of preptin on insulin secretion was therefore tested using a β-cell secretagogue protocol βTC6-F7 cells were subcultured at passage #52 into 24-well plates at 4 x 10^5 cells/well. They were grown for 3d in nDMEM to 80% confluence, then washed twice with KRB-HEPES. Stock preptin was serially diluted in incubation medium containing 10 mM D(+) glucose to yield final concentrations of 150, 75, 25, 5, 1 and 0.1 nM. Cells were then washed, and 1 ml/well of incubation medium containing 10 mM and various final preptin concentrations was added to each well. Cells were incubated at 37°C for 2 h, then medium removed. Cells were washed thrice with PBS, then lysed with 500 μl of lysis buffer. Incubation medium was centrifuged (16,000 x g, 3 min) and the supernatant separated from pelleted debris. Incubation medium and lysates were then analysed for insulin, preptin and protein as above.

Results

The results of the above are shown in Figures 1, 2 and 3.

Discussion

Mouse preptin is a 34 amino acid peptide which corresponds to Asp_{69}-Leu_{102} of murine proIGF-II E-peptide.

Preptin was present in granules at 1:8 the content of insulin, but 2:1 that of amylin (mol/mol), as determined by integration of RP-HPLC peak-areas. Preptin is flanked NH_2-terminally by a recognised Arg cleavage site, and COOH-terminally by a putative dibasic (Arg-Arg) cleavage motif (Bell (1984)) (Figure 1e). These residues likely serve as post-translational processing signals, and are highly conserved between species. Many prohormone precursors incorporate more than one hormone with differential proteolytic processing often being tissue specific (Martinez (1989)). The above results indicated that proIGF-II is a prohormone with more than one peptide-hormone product.

IGF-II is a member of the insulin family that regulates cell growth, differentiation and metabolism (De Chiara *et al* (1990). It is a single polypeptide chain derived from the

BCA and D domains of proIGF-II (see Figure 1e) and is widely synthesised in fetal and adult tissues. Insulin expression, on the other hand, is almost completely confined to β-cells. In mammalian genomes, the IGF-II gene is contiguous with those of insulin (Bell (1985)) and recent studies in humans have identified a VNTR polymorphism upstream of the *INS* and *IGF-II* genes, which may contribute to differential regulation of both genes (Ong (1999)).

The preptin radioimmunoassay (RIA) (Figure 2a) and reanalysis of the granule purification profiles of Figures 1a with the preptin RIA showed that preptin co-purified with insulin and amylin, confirming that it was indeed a granule component. Preptin-like immunoreactive material (PLIM) was characterised by RP-HPLC/RIA in purified granules and in βTC6-F7 conditioned medium. The major form of both intra-granular and extracellular PLIM co-eluted on RP-HPLC (Figure 2b). Mass spectrometry of HPLC-purified material corresponding to the PLIM peak from βTC6-F7 cells showed the presence of a single species, with molecular mass identical to that of murine preptin (Figure 2c). RP-HPLC also demonstrated that the major form of PLIM from human and rat plasma co-eluted with intragranular murine preptin. Preptin was co-secreted with insulin from βTC6-F7 cells in response to glucose-stimulation (Figure 2d), reaching maximal levels at 1-mM or greater.

These results confirm that preptin is synthesised in islet β-cells and packaged in secretory granules. Further, it is co-secreted with insulin in a glucose-dependent manner.

There is evidence that insulin secretion may be modulated by islet β-cell hormones, including insulin (Kulkarni (1999); Elahi (1982); Argoud (1987)), amylin (Waggoner *et al* (1993); Silvestre (1996); Degano *et al* (1993)), and pancreastatin (Tatemoto (1986)). These are thought to act through autocrine negative-feedback loops, mediated via binding to specific cell-surface receptors. The effects of preptin on insulin secretion were therefore investigated. The results obtained showed that synthetic rat preptin enhanced the glucose (10-mM)-stimulated secretion of insulin from cultured βTC6-F7 cells, in a manner that was both concentration-dependent and saturable (Figure 3a). Significant effects of preptin compared to controls (0 added preptin) were detected at concentrations of 0.1-nM and greater, and reached maximal at 75-nM. This concentration is equivalent to that at which amylin elicits inhibition of insulin secretion (Degano *et al* (1993)). These preptin concentrations are similar to those secreted from βTC6-F7 cells (Figure 2d), and are thus likely to occur adjacent to β-

cell membranes *in situ* in physiological islets. This demonstration of concentration-dependent and saturable stimulation of insulin secretion by preptin suggests that it elicits these effects by binding to a cell surface receptor.

The effect of infused synthetic rat preptin on glucose (20-mM)-stimulated insulin secretion in the isolated-perused rat pancrease (Figure 3b) was measured employing a maximally-effective preptin concentration (75-nM). Preptin significantly enhanced (by 30%; $p = 0.03$, 2-tailed *t*-test of areas-under-curve) the second phase of insulin secretion, compared with control values (0-added preptin) (Figure 3b). These findings are consistent with those obtained from βTC6-F7 cells (Figure 3a). They suggest that preptin is a physiological regulator of insulin secretion, which acts in a newly recognised feed-forward autocrine loop to enhance glucose-stimulated insulin secretion, and may function to counterbalance the inhibitory effects of other β-cell hormones on insulin secretion.

It is therefore the applicants view that preptin acts to recruit, prime and co-ordinate the glucose-responsive activity of β-cells in a local manner, amplifying the glucose-evoked signal to the β-cell organ. This action would be similar to the feed-forward mechanism effected in platelets by the thrombin-elicited release of thromboxane A_2 (Barritt (1992)).

The existence of a previously unsuspected mechanism, through which a new islet β-cell hormone amplifies glucose-mediated insulin secretion, suggests that preptin biology will be important in type 2 diabetes mellitus, which is characterised by a complex impairment of insulin secretion (De Fronzo *et al* (1992)). A defect in preptin synthesis, secretion, or action could contribute to the defective glucose-mediated insulin secretion in this condition and preptin administration may be advantageous for the treatment of type 2 diabetes mellitus or other disorders associated with diminished β-cell insulin secretion. It is noted that, in humans, the variable number of tandem repeat (VNTR) polymorphism upstream of the adjacent insulin (INS) and IGF-II genes regulates expression of both genes, and is associated with an increased tendency to both type 2 diabetes mellitus and polycystic ovary syndrome.

Section B

Preptin is co-packaged with insulin in islet tissue

To study preptin physiology, immunohistochemical studies were performed in normal murine pancreas using a preptin-specific antiserum. Synthetic rat preptin, prepared as above, (Auspep Pty Ltd) was conjugated to ovalbumin using the single-step gluteraldehyde method at pH 7.0 (Harlow and Lane). New Zealand white rabbits were used to raise polyclonal antisera against the rat preptin conjugate.

Serial sections from normal adult mouse (FVB/n) pancreas were stained with haematoxylin and specific anti-preptin or anti-insulin antisera, all at final dilutions of 1:40 (v/v), and with goat-anti-rabbit immunoperoxidase-labelled second antibody. Preptin (1 or 5 $mg.ml^{-1}$) was pre-incubated with anti-preptin antiserum for 30 min before addition to sections to demonstrate the specificity of preptin immunostaining.

Preptin-like immunoreactive material (PLIM) and insulin-like immunoreactive material were co-localised in islet β-cells (Figs. 4a,b). Competition studies showed that PLIM-staining was suppressed by pre-incubating preptin antiserum with synthetic preptin in a concentration dependent manner (Figs. 4b-d). These studies suggest that preptin is present in physiological pancreatic islet β-cells.

PLIM is present in normal islet tissue

To establish the identity of PLIM in normal islet tissue, we performed RP-HPLC/RIA of acid ethanol extracts from isolated rat islets. Pancreatic islets were isolated from normal adult male Wistar rats, and the contents extracted with acid ethanol according to a modification of published methods (Wollheim and Sharp (1981), Romanus (1988)).

The results are shown in Figure 5.

Although preptin levels were much lower than in βTC6-F7 cells, the major peak of PLIM co-eluted with intra-granular preptin, indicating that preptin is the dominant physiological component of PLIM in normal islets (Fig. 5). These data confirm that the preptin purified from the βTC6-F7 cells was not simply an artefact resulting from proteolysis during purification, but exists and is secreted in this form from both βTC6-F7 cells and normal rat islets.

Preptin is co-secreted with insulin in response to glucose stimulation.

Given the co-localisation of preptin and insulin within the β-cell secretory granule, experiments were undertaken to determine whether preptin and insulin are co-secreted in a regulated manner. Glucose-stimulated peptide secretion was studied according to published methods using both βTC6-F7 cells (Efrat *et al* (1993), Knaack *et al* (1994)) and isolated rat islets (Gotoh *et al* (1987)), and concentrations of preptin and insulin were measured using specific RIAs.

The results are shown in Figure 6. These indicated that while βTC6-F7 cells were responding to sub-physiological concentrations of glucose (<5 mM) (S Efrat, personal communication), a clear pattern of insulin/preptin co-secretion was observed from both βTC6-F7 cells (Fig. 6a) and normal rat islets (Fig. 6b). The amount of preptin secreted from the islet tissue (preptin:insulin 1:500) was much lower than the level secreted from the βTC6-F7 cells (preptin:insulin 1:8). This observation supported the HPLC/RIA results which indicated much lower levels of preptin in physiological tissue than in the cultured β-cells. Although preptin levels are much lower in physiological islets, both of these models clearly showed that preptin is co-secreted with insulin from physiological islet β-cells in response to glucose-stimulation.

Removal of endogenous preptin significantly decreases insulin secretion from the isolated-perfused rat pancreas.

To determine the role that endogenous pancreatic preptin might play in insulin secretion, the action of endogenous preptin was removed by infusing anti-preptin antibodies into the isolated perfused pancreas model as follows:

Pancreases were perfused with KHB supplemented with 4% dextran, 0.5% BSA, 3 mM-arginine and 5.5 mM glucose (final concentrations). Perfusate was gassed with a mixture of 95% O_2/5% CO_2 and infused by peristaltic pump at 2.7 ml.min^{-1} without re-circulation. Pancreases were perfused and equilibrated for 20-min prior to each 70-min perfusion. 10-min into the experiment either anti-preptin γ-globulin or non-immune rabbit γ-globulin were introduced via a side-arm infusion (final γ-globulin concentration in perfusate: 35 μg.ml^{-1} in carrier buffer (0.1% BSA in 0.9% NaCl). In addition, at 25-min, glucose was infused for 20-min (measured final concentration in perfusate: 20 mM). Continuous 1-min fractions were collected on ice and assayed for insulin (RIA).

Rabbit anti-rat preptin γ-globulin or control (non-immune rabbit) γ-globulin were purified by Protein A affinity chromatography (Pharmacia-Biotech, Hi-Trap Protein A *Tech. Rep.* (Wikstroms, Sweden (1999)) to diminish the potential influence from other serum constituents. The compositions of the two different γ-globulin fractions were confirmed by MALDI-TOF MS (Fig. 7a,b), and the binding capacities of the two different γ-globulin fractions were determined under conditions simulating the antibody perfusion experiments as above. The maximal amount of preptin completely bound by anti-preptin γ-globulin under the perfused pancreas experimental conditions was 20 ng/min (Fig. 7c).

Isolated perfused pancreases were infused with anti-preptin or control γ-globulin and subjected to square-wave stimulation by 20 mM glucose (Fig. 7d). Secretion of insulin in both the first and second phase was significantly decreased by anti-preptin γ-globulin (first phase: average 29% inhibition compared to controls, P = 0.02, 1-tailed *t*-test; second phase: average 26% inhibition compared to controls, *P*=0.03, 1-tailed *t*-test of AUC). In this experiment we have shown that removal of endogenous circulating preptin causes a significant decrease in glucose-mediated insulin secretion. This result is all the more interesting given that preptin has been estimated to be present in relatively low concentrations in the physiological islet (approximately 500x less than insulin) and yet still has the ability to exert a significant effect on insulin secretion. These experiments are consistent with the premise that physiological concentrations of pancreatic preptin play an autocrine role to increase glucose-mediated insulin secretion. This action may be similar to the feed-forward mechanism evoked in platelets by the thrombin-elicited release of thromboxane A_2 (Barrit (1992)).

Overall Conclusion

In summary, preptin is a previously unknown, pancreatic islet β-cell hormone. It is produced from the E-peptide of pro-IGF-II, is present in islet β-cell granules in significant amounts, is co-secreted with insulin in a regulated manner, enhances glucose-stimulated insulin secretion, and may act in a feed-forward autocrine loop, probably via binding to a β-cell surface receptor.

INDUSTRIAL APPLICATION

As described above, the present invention provides preptin (including in its human, rat and mouse forms) and analogs of preptin. Preptin and its analogs play a physiological role in the stimulation of glucose evoked insulin secretion.

The invention therefore also provides methods by which glucose-evoked insulin secretion can be modulated. Such modulation will usually involve administration of preptin and its analogs as described above. However, modulation can also be achieved by use of preptin agonists and antagonists.

A preptin agonist is a compound which promotes or potentiates the effect of preptin on insulin secretion. In contrast, a preptin antagonist is a compound which competes with preptin or otherwise interacts with preptin to block or reduce the effect of preptin on insulin secretion.

Preptin agonists and preptin antagonists can be identified by assay systems which measure the effect preptin has on insulin secretion in the presence and absence of a test compound. For example, the assay systems described in the experimental section herein can be used.

Where it is desired that a preptin agonist or preptin antagonist be employed in modulating insulin secretion, the agonist/antagonist can be administered as a pure compound or formulated as a pharmaceutical composition as described above for preptin.

Also provided herein are immunological reagents which bind preptin. Such reagents (which can be polyclonal antibodies) can be generated using art standard techniques, including those described in the experimental section.

Monoclonal antibodies can also be provided. Such antibodies will typically be made by standard procedures as described, eg. in Harlow and Lane 1988. Briefly, appropriate animals are selected and the desired immunisation protocol followed. After the appropriate period of time, the spleens of such animals are excised and individual spleen cells fused, typically, to immortalised myeloma cells under appropriate selection conditions. Thereafter, the cells are clonally separated and the supernatants of each clone tested for the production of an appropriate antibody specific for the desired region of the immunising antigen.

Other suitable techniques for preparing antibodies involve *in vitro* exposure of lymphocytes to the antigen or alternatively, to selection of libraries of antibodies in phage or similar vectors. See, for example Huse *et al* 1989.

Also, recombinant antibodies may be produced using procedures known in the art. See, for example, US Patent 4,816,567.

The antibodies may be used with or without modification. Frequently, antibodies will be labelled by joining, either covalently or non-covalently a substance which provides a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in the literature.

Antibodies as above to preptin can therefore be used to monitor the presence of preptin in a patient or in preptin quantification assays. In such assays, any convenient immunological format can be employed. Such formats include immunohistochemical assays, RIA, IRMA and ELISA assays.

The assays can be conducted in relation to any biological fluid which does, or should, contain preptin. Such fluids include blood, serum, plasma, urine and cerebrospinal fluid.

The antibodies can also be included in assay kits. Such kits can contain, in addition, a number of optional but conventional components, the selection of which will be routine to the art skilled worker. Such additional components will however generally include a preptin reference standard, which may be preptin itself or an analog (such as a fragment).

It will also be appreciated that antibodies such as described above can, if some circumstances, also function as preptin antagonists by binding to preptin and partly or completely interfering with preptin activity.

As alluded to above, the applicants findings in respect of preptin also have diagnostic implications. For example, individuals whose preptin production is less than is required in order to elicit insulin secretion at appropriate levels, or who produce preptin in a less active or inactive (mutant) form will require therapeutic intervention. Diagnostic or prognostic methods are therefore within the scope of the invention.

In one specific embodiment, a diagnostic or prognostic method will involve detection of mutations in the gene coding for preptin and/or the preptin secretory mechanism. Detection can occur using any one of a number of art standard techniques including Single Stranded Confirmation Analysis (Orita *et al* (1989)) or the Amplification Refractory Mutation System (ARMS) as disclosed in European Patent Application Publication No 0 332 435.

If a mutation is detected, corrective approaches become possible. These include but are not limited to gene therapy. Again, art standard techniques will be employed.

Other implications and applications of the applicants identification of preptin will be apparent to those persons skilled in the art, who will appreciate that the above description is provided by way of example only and that the invention is not limited thereto.

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CLAIMS:

1. An isolated bioactive peptide having preptin functionality.

2. A bioactive peptide, the amino acid sequence of which is as follows:

Asp Val Ser Thr R_1 R_2 R_3 Val Leu Pro Asp R_4 Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe R_5 R_6 Asp Thr Trp R_7 Gln Ser R_8 R_9 Arg Leu

wherein:

R_1 is Ser or Pro;
R_2 is Gln or Pro;
R_3 is Ala or Thr;
R_4 is Asp or Asn;
R_5 is Gln or Lys;
R_6 is Tyr or Phe;
R_7 is Arg or Lys;
R_8 is Ala or Thr; and
R_9 is Gly or Gln,

or an analog thereof.

3. Human preptin having the amino acid sequence:

Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr Trp Lys Gln Ser Thr Gln Arg Leu,

or an analog thereof.

4. Rat preptin having the amino acid sequence:

Asp Val Ser Thr Ser Gln Ala Val Leu Pro Asp Asp Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Lys Phe Asp Thr Trp Arg Gln Ser Ala Gly Arg Leu,

or an analog thereof.

5. Mouse preptin having the amino acid sequence:

Asp Val Ser Thr Ser Gln Ala Val Leu Pro Asp Asp Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr Trp Arg Gln Ser Ala Gly Arg Leu,

or an analog thereof.

6. A mammalian homologue to human, rat or mouse preptin as defined in any one of claims 3 to 5.

7. A preptin analog which includes from 6 to 33 amino acids from a sequence as defined in any one of claims 2 to 5, and which retains preptin functionality.

8. A preptin analog which is, or includes, a hexapeptide, a heptapeptide, an octapeptide, a nonapeptide or a decapeptide derived from human preptin as defined in claim 3.

9. A peptide selected from human preptin having the amino acid sequence:

Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr Trp Lys Gln Ser Thr Gln Arg Leu,

or an analog thereof, wherein said analog is selected from the following:

(i) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr Trp Lys Gln Ser Thr Gln Arg;

(ii) Asp Val Ser Thr Pro Pro Thr Val Leu.Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr Trp Lys Gln Ser Thr Gln;

(iii) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr Trp Lys Gln Ser Thr;

(iv) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr Trp Lys Gln Ser;

(v) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr Trp Lys Gln;

(vi) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr Trp Lys;

(vii) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr Trp;

(viii) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr;

(ix) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp;

(x) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr;

(xi) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln;

(xii) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe Phe;

(xiii) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys Phe;

(xiv) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly Lys;

(xv) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val Gly;

(xvi) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro Val;

(xvii) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr Pro;

(xviii) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg Tyr;

(xix) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro Arg;

(xx) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro;

(xxi) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe;

(xxii) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn;

(xxiii) Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp;

(xxiv) Asp Val Ser Thr Pro Pro Thr Val Leu Pro;

(xxv) Asp Val Ser Thr Pro Pro Thr Val Leu;

(xxvi) Asp Val Ser Thr Pro Pro Thr Val;

(xxvii) Asp Val Ser Thr Pro Pro Thr; and

(xxviii) Asp Val Ser Thr Pro Pro.

10. An isolated polynucleotide which encodes preptin or an analog thereof as defined in any one of claims 1 to 9.

11. An isolated polynucleotide which encodes human preptin and which comprises the following nucleotide sequence:

gacgtgtcgacccctccgaccgtgcttccggacaacttccccagataccccgtgggcaagttcttccaatatga
cacctggaagcagtccacccagcgcctg.

12. An isolated polynucleotide which encodes rat preptin and which comprises the following nucleotide sequence:

gacgtgtctacctctcaggccgtacttccggacgacttccccagataccccgtgggcaagttcttcaaattcgac
acctggagacagtccgcgggacgcctg.

13. An isolated polynucleotide which encodes mouse preptin and which comprises the following nucleotide sequence:

gacgtgtctacctctcaggccgtacttccggacgacttccccagataccccgtgggcaagttcttccaatatgac
acctggagacagtccgcgggacgcctg.

14. A vector or cell line which includes a polynucleotide having the nucleotide sequence of any one of claims 11 to 13 and which is capable of expressing a peptide having preptin functionality.

15. A vector or cell line as defined in claim 14 which includes the nucleotide sequence of claim 11.

16. A pharmaceutical composition which comprises preptin or an analog thereof as defined in any one of claims 1 to 9.

17. A dosage form comprising a mammalian preptin or an analog thereof as defined in any one of claims 1 to 9 in combination with a physiological buffer solution suitable for administration to humans.

18. A dosage form as defined in claim 17 which is for administration by injection.

19. A dosage form as defined in claim 17 or claim 18 in which said preptin is human preptin or an analog thereof as defined in any one of claims 3, 8 and 9.

20. A preparation of a mammalian preptin or analog as defined in any one of claims 1 to 9 in which said preptin or analog is present in an amount of at least 50% by weight.

21. A preparation as defined in claim 20 in which said preptin or analog is at least 80% by weight of said preparation.

22. A preparation as defined in claim 20 in which said preptin or analog is at least 90% by weight of said preparation.

23. A preparation as defined in claim 20 in which said preptin or analog is at least 95% by weight of said preparation.

24. A preparation as defined in claim 20 in which said preptin or analog is at least 99% by weight of said preparation.

25. A preparation as defined in claim 20 in which said preptin or analog is substantially pure.

26. A preparation as defined in claim 20 in which said preptin or analog is pure.

27. A preparation as defined in claim 20 in which said preptin or analog is human preptin or an analog thereof.

28. A salt of a mammalian preptin or analog as defined in any one of claims 1 to 9.

29. A salt as defined in claim 28 which is a physiologically acceptable salt.

30. A salt as defined in claim 29 in which said preptin or analog is formed by combination with anions of an organic acid.

31. A salt as defined in claim 30 in which said salt is selected from malate, acetate, propionate, butyrate, oxaloacetate, citrate, isocitrate, α-ketoglutarate, succinate, fumarate and trifluoroacetate salts.

32. A pharmaceutical composition which includes a salt as defined in any one of claims 29 to 31.

33. A method of therapeutically or prophylactically treating a patient which comprises the step of administering to said patient an effective amount of preptin or an analog thereof as defined in any one of claims 1 to 9 or of a salt as defined in any one of claims 29 to 31.

34. A method of stimulating insulin secretion for a therapeutic or prophylactic purpose which comprises the step of administering to a patient in need of such therapy or prophylaxis an effective amount of preptin or an analog thereof as defined in any one of claims 1 to 9, or of a salt as defined in any one of claims 29 to 31.

35. A method of treating Type 2 diabetes mellitus which comprises the step of administering to a patient an effective amount of preptin or an analog thereof as defined in any one of claims 1 to 9, or of a salt as defined in any one of claims 29 to 31.

36. A method of treating a condition which results in or involves deficient insulin synthesis, secretion or action which comprises the step of administering to a

patient an effective amount of preptin or an analog thereof as defined in any one of claims 1 to 9, or of a salt as defined in any one of claims 29 to 31.

37. The use of preptin or an analog thereof as defined in any one of claims 1 to 9 in the preparation of a medicament.

38. The use of preptin or an analog thereof as defined in any one of claims 1 to 9 in the preparation of a medicament for stimulating insulin secretion.

39. The use of a salt as defined in any one of claims 29 to 31 in the preparation of a medicament.

40. The use of a salt as defined in any one of claims 29 to 31 in the preparation of a medicament for stimulating insulin secretion.

41. Antibodies which bind preptin or an analog thereof as defined in any one of claims 1 to 9.

42. A monoclonal antibody which binds preptin or an analog thereof as defined in any one of claims 1 to 9.

43. A monoclonal antibody which binds human preptin or an analog thereof as defined in any one of claims 3, 8 and 9.

44. An immunological assay which employs an antibody as defined in any one of claims 41 to 43.

45. An assay as defined in claim 44 in which the presence of preptin is quantitatively measured in a biological fluid.

46. An assay as defined in claim 45 in which the biological fluid is blood, serum, plasma, urine or cerebrospinal fluid (CSF).

47. An immunological assay which employs an antibody as defined in any one of claims 41 to 43 and which is a RIA, IRMA or ELISA.

48. An assay kit which includes an antibody as defined in any one of claims 41 to 43.

49. An assay kit as defined in claim 48 which comprises an antibody as defined in any one of claims 41 to 43 and a preptin reference standard.

50. An assay kit as defined in claim 49 in which said reference standard is preptin or an analog thereof as defined in any one of claims 1 to 9.

51. A method of identifying a preptin agonist which comprises the steps of:

testing the degree of insulin secretion induced by a pre-determined concentration of preptin as defined in claim 1 in the presence and absence of a candidate agonist; and

identifying as an agonist any compound which effects an increase in preptin-mediated insulin secretion.

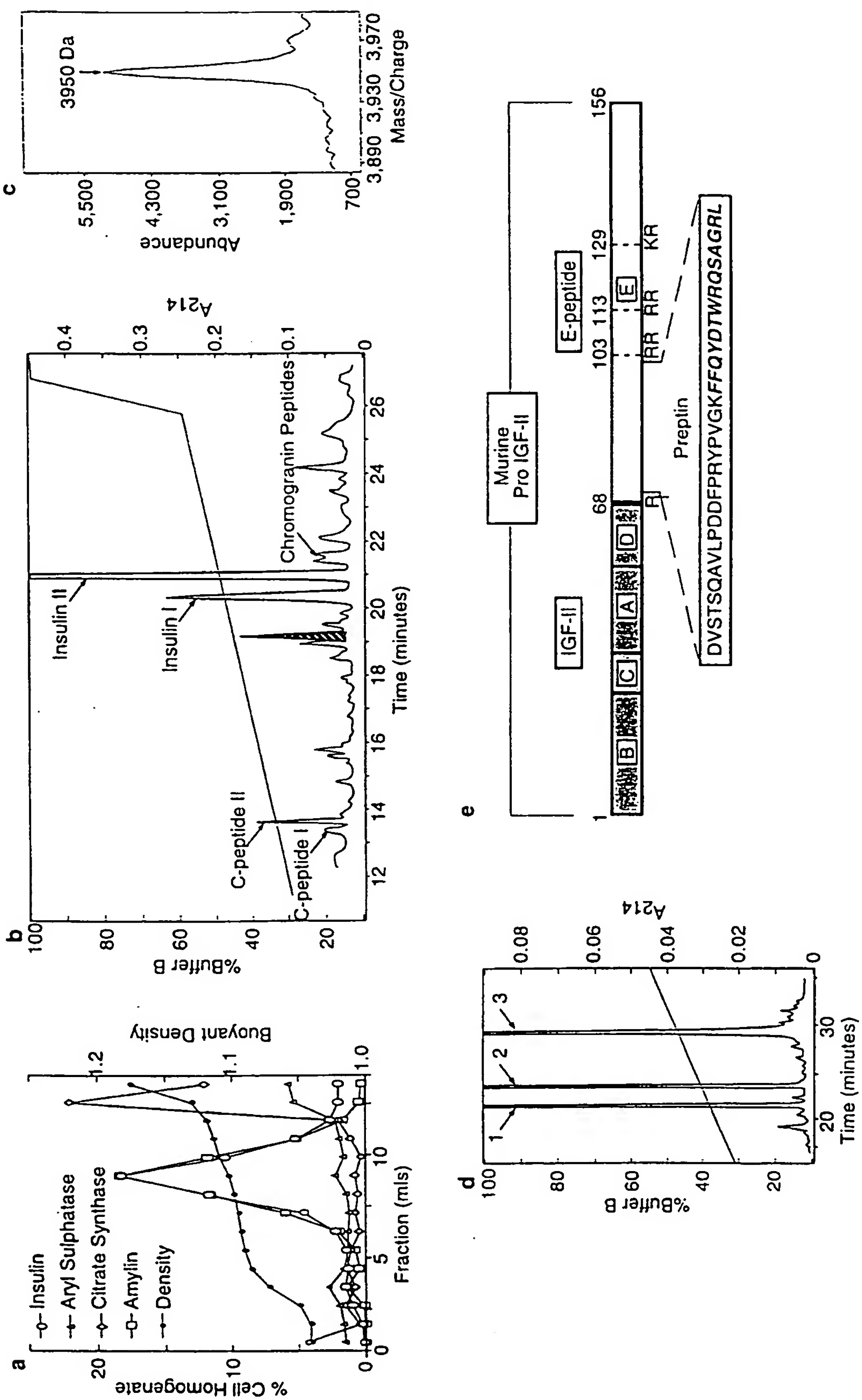
52. A method of identifying a preptin antagonist which comprises the steps of:

testing the degree of insulin secretion induced by a pre-determined concentration of preptin as defined in claim 1 in the presence and absence of a candidate antagonist; and

identifying as an antagonist any compound which effects a decrease in preptin-mediated insulin secretion.

53. A method of modulating glucose mediated insulin secretion which comprises the step of administering to a patient an effective amount of a preptin agonist or a preptin antagonist.

Figure 1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Fraction (mls)
Buoyant Density
Insulin
Aryl Sulphatase
Citrate Synthase
Amylin
Density
b
%Buffer B
A214
Time (minutes)
C-peptide II
C-peptide I
Insulin II
Insulin I
Chromogranin Peptides
c
Abundance
Mass/Charge
3950 Da
d
%Buffer B
A214
Time (minutes)
1
2
3
e
Murine Pro IGF-II
IGF-II
E-peptide
Preptin
B
C
A
D
E
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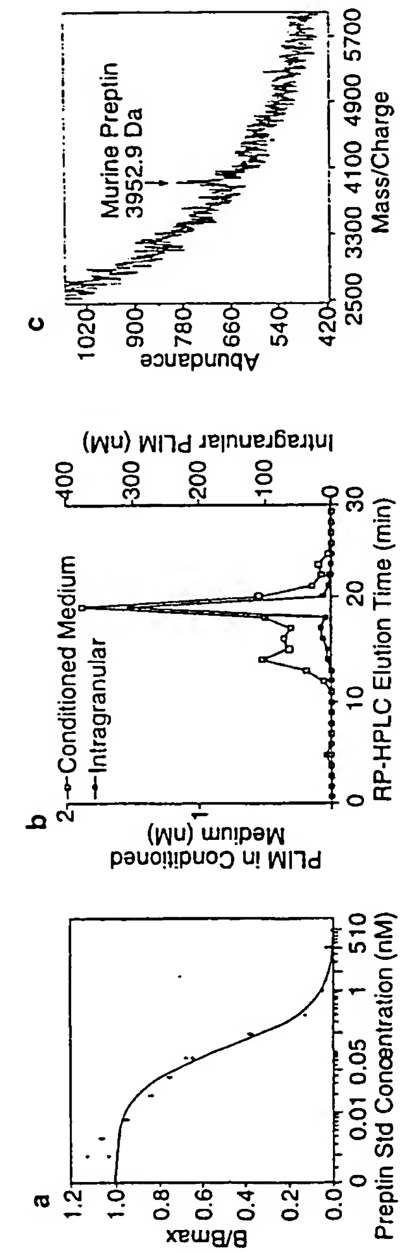

a
B/Bmax
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
0.01
0.05
1
510
Preptin Std Concentration (nM)
b
PLIM in Conditioned Medium (nM)
2
1
0
10
20
30
RP-HPLC Elution Time (min)
Intragranular PLIM (nM)
400
300
200
100
Conditioned Medium
Intragranular
c
Abundance
1020
900
780
660
540
420
2500
3300
4100
4900
5700
Mass/Charge
Murine Preptin
3952.9 Da

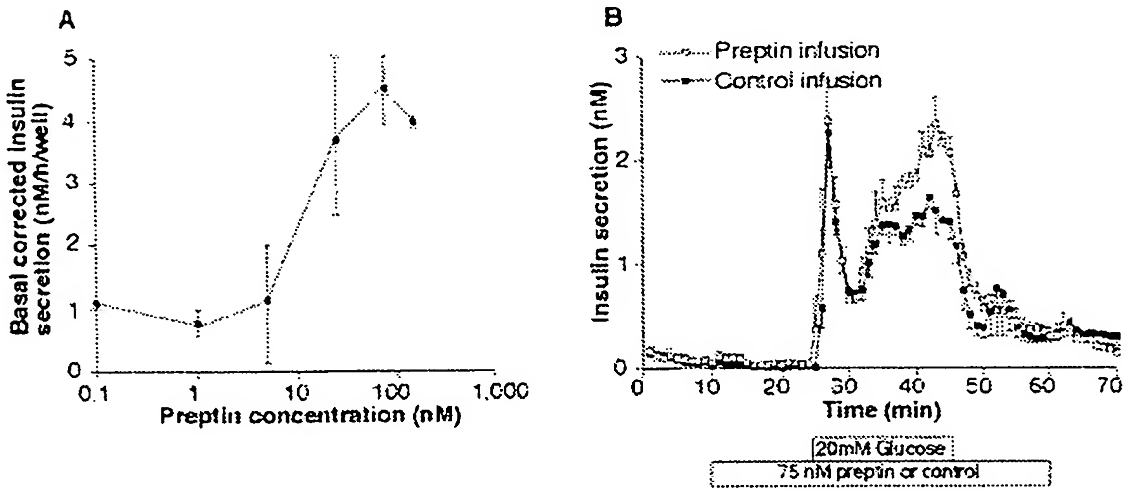
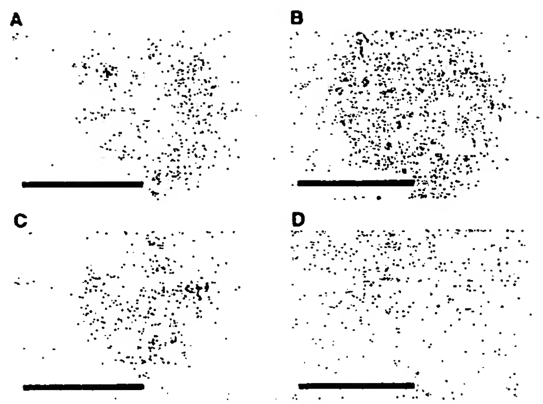

A
Basal corrected insulin secretion (nM/h/well)
0
1
2
3
4
5
0.1
1
10
100
1,000
Preptin concentration (nM)
B
Preptin infusion
Control infusion
Insulin secretion (nM)
0
1
2
3
0
10
20
30
40
50
60
70
Time (min)
20mM Glucose
75 nM preptin or control

FIGURE 3



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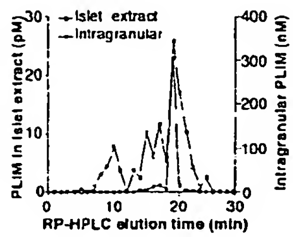
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Intragranular
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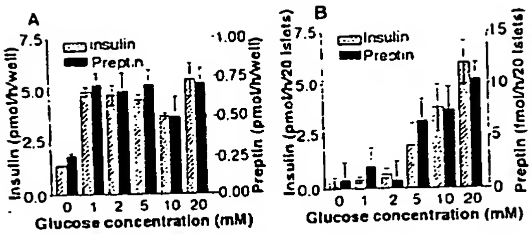
Intragranular PLIM (nM)
RP-HPLC elution time (min)

FIGURE 5



A
Insulin
Preptin
Insulin (pmol/h/well)
Preptin (pmol/h/well)
Glucose concentration (mM)
B
Insulin
Preptin
Insulin (pmol/h/20 islets)
Preptin (fmol/h/20 islets)
Glucose concentration (mM)

FIGURE 6

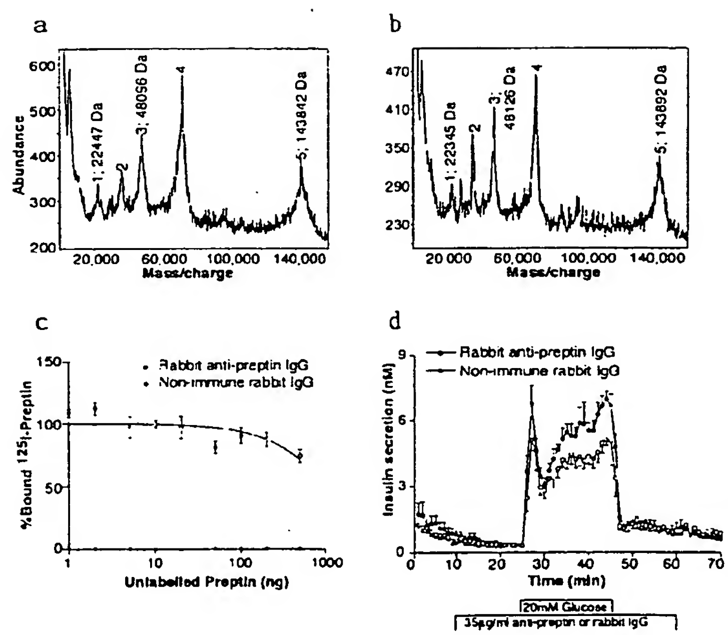

a
Abundance
Mass/charge
1; 22447 Da
2
3; 48056 Da
4
5; 143842 Da
b
1; 22345 Da
2
3; 48126 Da
4
5; 143892 Da
c
Rabbit anti-preptin IgG
Non-immune rabbit IgG
%Bound 125I-Preptin
Unlabelled Preptin (ng)
d
Rabbit anti-preptin IgG
Non-immune rabbit IgG
Insulin secretion (nM)
Time (min)
20mM Glucose
35µg/ml anti-preptin or rabbit IgG

FIGURE 7

SEQUENCE LISTING

<110> Cooper, Garth J S
Buchanan, Christina M

<120> Peptide

<130> 26010 MRB

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<150> NZ336359
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1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ00/00102

A.	**CLASSIFICATION OF SUBJECT MATTER**

Int. Cl. [7]: C07K 14/47, 7/06, 7/04, 16/18. A61K 38/17, 38/08, 38/10, A61P 5/50, C12N 15/12, G01N 33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B.	**FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN: Peptide sequence search.
STN: (CA, MEDLINE, WPIDS, BIOSIS) Keywords: Preptin, Pancreatic, Islets, Beta, Peptide, Protein

C.	**DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AU 13286/92 (641855) B (Amylin Pharmaceuticals, Inc.) 23 July 1992. Page 10 line 14 to page 13 line 27	1
X	AU 59537/90 (620727) B (Amylin Corporation) 6 February 1991. Page 1 line 26 to page 2 line 26	1
X	AU 29494/89 (631112) B (Amylin Corporation) 1 August 1989. Page 14 lines 6-10	1

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

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"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 September 2000	04 OCT 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer J.G. HANSON Telephone No : (02) 6283 2262

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ00/00102

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AU 21575/88 (634954) B (Amylin Corporation) 27 July 1989. Page 4 lines 2-6	1
X	CHEMCATS Acc. No. (AN) 1998:22309, Advanced ChemTech Product Catalog (10 June 1999) CAS Reg. No. (RN) 198757-90-3 See Abstract	1-3, 6-11, 14-53

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/NZ00/00102

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	13286/92	CA	2076658	CA	2077265	EP	525149
		EP	525158	SG	43866	US	5234906
		WO	9211862	WO	9211863	ZA	9200005
		US	5321008	US	5508260	US	5527771
		US	6048514	AU	16873/92	AU	20115/92
		AU	21693/92	AU	61964/96	CA	2082928
		CA	2109604	CA	2109794	EP	533898
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		SG	44838	SG	49239	WO	9216222
		WO	9220367	WO	9220366	US	5814600
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AU	59537/90	CA	2020786	EP	408294	NO	910901
		NZ	234428	WO	9100737	US	5280014
		US	5364841	AU	29494/89	DK	4438/89
		EP	348490	FI	950530	HK	1002990
		JP	10267914	NO	893606	NO	951250
		NZ	227601	WO	8906135	US	5266561
		US	5281581	US	5716619	US	5942227
AU	21575/88	DK	4738/88	EP	309100	EP	787741
		FI	883936	HK	1000934	JP	1096137
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		HK	1003033	JP	1063594	JP	9133679
		JP	10168099				

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